APPLICATION

FOR

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FOR

NITRIC OXIDE-PRODUCING HYDROGEL MATERIALS

NITRIC OXIDE-PRODUCING HYDROGEL MATERIALS

Field of the Invention

The present invention relates to *polymerizable* hydrogel

materials that produce physiologically relevant amounts of nitric oxide (NO)

for prolonged periods of time.

This application claims priority to U.S.S.N. 60/152,054 filed

September 2, 1999.

Background of the Invention

Endothelial cells, normally present as a monolayer in the intimal

layer of the arterial wall, are believed to play an important role in the

regulation of smooth muscle cell *(smc)* proliferation *in vivo*. Endothelial cells are

seriously disrupted by most forms of vascular injury, including that caused

by percutaneous transluminal coronary angioplasty and similar procedures.

Approximately 35-50% of patients treated by percutaneous transluminal

coronary angioplasty experience clinically significant renarrowing of the

artery, or restenosis, within six months of the initial treatment. Restenosis is

due, at least in part, to migration and proliferation of smooth muscle cells in

the arterial wall along with increases in secretion of matrix proteins to form

an obstructive neointimal layer within the arterial wall. Similar issues limit

the performance of vascular grafts. The processes that regulate arterial

wound healing following vascular injury, such as that caused by angioplasty,

are as yet poorly understood, but are believed to involve a complex cascade

of blood and vessel wall-derived factors.

Numerous factors that stimulate intimal thickening and restenosis

have been identified through administration of exogenous proteins, genetic

alteration of cells, or through the blockade of certain signals using antibodies

or other specific growth factor inhibitors. These smooth muscle cell

mitogens and chemoattractants derive from both the blood or thrombus

formation and from the vessel wall itself. Endothelial cells produce a

number of substances known to down-regulate smooth muscle cell

proliferation, including heparin sulfate, prostacyclin (PG12), and NO.

NO is an endothelium-derived target molecule useful for the prevention of restenosis because, in addition to limiting the proliferation of smooth muscle cells (Garg et al., 1989), NO reduces platelet aggregation (de

5   Graaf et al., 1992; Radomski et al., 1987), increases endothelial cell proliferation (Ziche et al., 1993), and attenuates leukocyte adhesion (Lefer et al., 1993), all of which are highly desirable for the reduction of intimal thickening and restenosis (Reviewed by Loscalzo, 1996). Because of the complexity of the restenotic process, approaches that act upon multiple

10   targets are the most likely to be successful.

The mechanisms whereby NO affects these multiple responses are not fully understood as yet, but it is known that NO activates soluble guanylate cyclase by binding to its heme moiety, thereby elevating the levels of cyclic guanosine monophosphate (cGMP), an intracellular second

15   messenger with multiple cellular effects (Moro et al., 1996). The effects of NO can often be mimicked by the administration of cGMP or more stable derivatives of cGMP (Garg et al., 1989). In addition, NO has been found to inhibit ribonucleotide reductase, an enzyme that converts ribonucleotides into deoxy ribonucleotides, thus significantly impacting DNA synthesis

20   (Lepoivre et al., 1991; Kwon et al., 1991), as well as several enzymes involved in cellular respiration (Stuehr et al., 1989).

A number of molecules that produce NO under physiological conditions (NO donors) have been identified and evaluated both *in vitro* and *in vivo*. NO donor molecules exert biological effects mimicking those of NO

25   and include S-nitrosothiols (Diodati et al., 1993; Lefer et al., 1993; DeMeyer et al., 1995), organic nitrates (Ignarro et al., 1981), and complexes of NO with nucleophiles (Diodati et al., 1993; Diodati et al., 1993; Maragos et al., 1993). Most of these have been low molecular weight molecules that are administered systemically and have short half-lives under physiologic

30   conditions, thus exerting effects upon numerous tissue types with a brief period of activity. In addition, L-arginine is often thought of as a NO donor, as L-arginine is a substrate for NO synthase, and thus administration of L-

arginine increases endogenous NO production and elicits responses similar to those caused by NO donors in most cases (Cooke et al., 1992, *et, 9/12*)

The development of NO-releasing polymers containing NO/nucleophile complexes has been reported by Smith et al., (1996). These
5 materials were capable of releasing NO for as long as 5 weeks *in vitro* and were able to limit smooth muscle cell proliferation in culture and to reduce platelet adherence to vascular graft materials in an arterio-venous shunt model. These materials show promise for numerous clinical applications where localized NO production would be desired, such as anti-thrombotic
10 coating materials for catheters, but probably will not be useful for the direct treatment of tissues *in vivo* as these materials suffer from a number of disadvantages. These polymers may be produced as films, powders, or microspheres, but they cannot be formed *in situ* in direct contact with cells and tissues, thus making it difficult to strictly localize NO treatment to a
15 tissue and potentially causing issues with the retention of the polymer at the site of application. The formulation issues will also make local administration during laparoscopic or catheter-based procedures difficult or impossible. Additionally, biocompatibility of the base polymer is a serious issue for implantable, NO-releasing polymers, especially those intended for
20 long-term use, as inflammatory and thrombotic responses may develop after the cessation of NO release.

It would be more efficient if these compounds could be administered solely to the site in need of treatment, and in some cases, reduce or eliminate side effects due to systemic administration of the agents, particularly over
25 prolonged time periods.

It is therefore an object of the present invention to provide reagents for controlled release of NO and/or compounds modulating NO levels at a particular site, following local or topical application.

It is a further object of the present invention to provide methods for
30 treatment of conditions involving inflammatory responses by providing hydrogel materials releasing compound modulating NO levels at the site of application.

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Summary of the Invention

Biocompatible polymeric materials releasing or producing NO, most
preferably photopolymerizable biodegradable hydrogels capable of releasing
physiological amounts of NO for prolonged periods of time, are applied to

5   sites on or in a patient in need of treatment thereof for disorders such as
restenosis, thrombosis, asthma, wound healing, arthritis, penile erectile
dysfunction or other conditions where NO plays a significant role. The
polymeric materials can also be formed into films, coatings, or
microparticles. The polymers are typically formed of macromers, which

10   preferably include biodegradable regions, and have bound thereto groups that
are released *in situ* to elevate or otherwise modulate NO levels at the site
where treatment is needed. The macromers can form a homo or hetero-
dispersion or solution, which is polymerized to form a polymeric material,
that in the latter case can be a semi-interpenetrating network or

15   interpenetrating network. Compounds to be released can be physically
entrapped, covalently or ionically bound to macromer, or actually form a part
of the polymeric material. Hydrogels can be formed by ionic and/or covalent
crosslinking. Other active agents, including therapeutic, prophylactic, or
diagnostic agents, can also be included within the polymeric material.

Brief Description of the Drawings

20   Figure 1 is a schematic of the synthesis of S-nitrosocysteine
hydrogels (Acryloyl-PEG-CYS-NO).

Figure 2 is a schematic of the synthesis of acryloyl-PEG-Lysine$_5$ NO-
nucleophile complex hydrogels.

25   Figure 3 is a schematic of the synthesis of acryloyl-PEG-DETA-NO-
nucleophile complex hydrogels.

Figure 4 is a graph showing the temporal release (%NO released over
time in days) of NO from acryloyl-PEG-Lys$_5$-NO hydrogels at pH 7.4
(circles) and pH 3 (squares).

30   Figure 5 is a graph showing the temporal release (%NO released over
time in hours) of NO from acryloyl-PEG-DETA-NO hydrogels at pH 7.4
(circles) and pH 2 (squares).

Figure 6 is a graph showing the temporal release (%NO released over time in hours) of NO from PEG-~~CYSNO~~ *Cys-NO* hydrogels at pH 7.4 (circles) and pH 2 (squares). *A*

Figure 7 is a graph showing the temporal release (µmol NO released per gram of polymer over time in hours) of NO from PVA-NO-bFGF hydrogels at pH 7.4, 37°C. *Arya 14*

Figures 8A and 8B are graphs showing that acryloyl-PEG-Lysine-NO hydrogels inhibit the proliferation of smooth muscle cells. Figure 8A, % of control cell number, hydrogel formulation. Figure 8B, % of control cell number, soluble polymer.

Figures 9A and 9B are graphs showing the inhibition of SMC proliferation by NO released from acryloyl-PEG-DETA-NO hydrogels (Figure 9A) and soluble polymer (Figure 9B), as a percentage of the control.

Figures 10A and 10B are graphs showing inhibition of SMC proliferation by NO released from acryloyl-PEG-~~CYSNO~~ *Cys-NO* hydrogels (Figure 10A) and soluble polymer (Figure 10B), as a percentage of controls.

Figure 11 is a graph comparing the degree of inhibition of smooth muscle cell growth by NO released from hydrogels: acryloyl-PEG-Lys-NO, acryloyl-PEG-DETA-NO, and acryloyl-PEG-~~CYSNO~~ *Cys-NO*, compared to control hydrogel with NO. The %inhibition of smooth muscle cell growth is determined by comparing the cell growth for each NO-releasing hydrogel to a control PEG-diacrylate hydrogel.

~~Figure 12a is a graph showing the temporal release of NO, micromolar NO released/gram of gel over time in hours from PVA-NO-bFGF hydrogels at pH 7.4, 37°C.~~ Figure 12b is a graph showing the temporal ~~release (% of theoretical bFGF released per gram of gel over time in hours) from PVA-Cys-NO-bFGF hydrogels at pH 7.4, 37°C.~~

Detailed Description of the Invention

I.   Polymeric Materials for Release of NO

The polymeric materials are biocompatible and release or produce NO. In various preferred embodiments, the polymers are also biodegradable, form hydrogels, polymerize *in situ* and are tissue ~~adherent~~ *adhesive*. These properties

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are conferred by the selection of the macromer components as well as addition of various groups to the components.

The term "polymerizable" means that the regions have the capacity to form additional covalent bonds resulting in macromer interlinking, for example, carbon-carbon double bonds of acrylate-type molecules. Such polymerization is characteristically initiated by free-radical formation resulting from photon absorption of certain dyes and chemical compounds to ultimately produce free-radicals, although it can be obtained using other methods and reagents known to those skilled in the art.

A.   Polymeric Materials

The polymeric materials must be biocompatible, i.e., not eliciting a significant or unacceptable toxic or immunogenic response following administration to or implantation into an individual.

A number of polymeric materials are known which are biocompatible, including both natural and synthetic polymers. Examples include proteins (of the same origin as the recipient), polysaccharides such as chondroitin sulfate and hyaluronic acid, polyurethanes, polyesters, polyamides, and acrylates. Polymers can be degradable or non-degradable.

Most polymeric materials will be selected based on a combination of properties conferred by the various components, which may include a water soluble regions such as PEG or PVA, biodegradable regions such as regions that degrade hydrolytically, and groups that can be used to polymerize the macromers *in situ*.

Water-Soluble and/or Tissue Adhesive Regions

There are a variety of water soluble materials that can be incorporated into the polymers. The term "at least substantially water soluble" is indicative that the solubility should be at least about 5 g/100 ml of aqueous solution. In preferred embodiments, the core water soluble region can consist of poly(ethylene glycol), poly(ethylene oxide), poly(vinyl acetate), poly(vinyl alcohol), poly(vinylpyrrolidone), poly(ethyloxazoline), poly(ethylene oxide)-co-poly(propyleneoxide) block copolymers, polysaccharides or carbohydrates such as hyaluronic acid, dextran, heparin

sulfate, chondroitin sulfate, heparin, or alginate, or proteins such as gelatin, collagen, albumin, or ovalbumin.

Hydrophilic (i.e., water soluble) regions will generally be tissue adhesive. Both hydrophobic and hydrophilic polymer including large

5 number of exposed carboxylic groups will be tissue or bioadhesive. Ligands such as RGD peptides and lectins which bind to carbohydrate molecules on cells can also be bound to the polymer to increase tissue adhesiveness.

Degradable Regions

Polyesters (Holland *et al.*, 1986 *Controlled Release*, 4:155-180) of α-

10 hydroxy acids (*viz.*, lactic acid, glycolic acid), are the most widely used biodegradable materials for applications ranging from closure devices (sutures and staples) to drug delivery systems (U.S. Patent No. 4,741,337 to Smith *et al.*; Spilizewski *et al.*, 1985 *J. Control. Rel.* 2:197-203). In addition to the poly(hydroxy acids), several other polymers are known to biodegrade,

15 including polyanhydrides and polyorthoesters, which take advantage of labile backbone linkages, as reported by Domb *et al.*, 1989 *Macromolecules*, 22:3200; Heller *et al.*, 1990 *Biodegradable Polymers as Drug Delivery Systems*, Chasin, M. and Langer, R., Eds., Dekker, New York, 121-161. Polyaminoacids have also been synthesized since it is desirable to have

20 polymers that degrade into naturally occurring materials, as reported by Miyake *et al.*, 1974, for *in vivo* use.

The time required for a polymer to degrade can be tailored by selecting appropriate monomers. Differences in crystallinity also alter degradation rates. Due to the relatively hydrophobic nature of these

25 polymers, actual mass loss only begins when the oligomeric fragments are small enough to be water soluble. Hence, initial polymer molecular weight influences the degradation rate.

The biodegradable region is preferably hydrolyzable under *in vivo* conditions. Hydrolyzable groups may be polymers and oligomers of

30 glycolide, lactide, ε-caprolactone, other α-hydroxy acids, and other biologically degradable polymers that yield materials that are non-toxic or

present as normal metabolites in the body. Preferred poly(α-hydroxy acid)s are poly(glycolic acid), poly(DL-lactic acid) and poly(L-lactic acid). Other useful materials include poly(amino acids), poly(anhydrides), poly(orthoesters), and poly(phosphoesters). Polylactones such as poly(ε-

5   caprolactone), poly(ε-caprolactone), poly(δ-valerolactone) and poly(gamma-butyrolactone), for example, are also useful.

Biodegradable regions can also be constructed from polymers or monomers using linkages susceptible to biodegradation by enzymes, such as ester, peptide, anhydride, orthoester, and phosphoester bonds. Degradable

10   materials of biological origin are well known, for example, crosslinked gelatin. Hyaluronic acid has been crosslinked and used as a degradable swelling polymer for biomedical applications (U.S. Patent No. 4,987,744 to della Valle et al., U.S. Patent 4,957,744 to Della Valle *et al.* (1991) *Polym. Mater. Sci. Eng.*, 62:731-735]).

15   *Biodegradable Hydrogels*

A number of polymers have been described which include both water soluble regions and biodegradable regions. Sawhney et al., (1990) *J. Biomed. Mater. Res.* 24:1397-1411, copolymerized lactide, glycolide and ε-caprolactone with PEG to increase its hydrophilicity and degradation rate.

20   U.S. Patent No. 4,716,203 to Casey et al. (1987) synthesized a PGA-PEG-PGA block copolymer, with PEG content ranging from 5-25% by mass. U.S. Patent No. 4,716,203 to Casey et al. (1987) also reports synthesis of PGA-PEG diblock copolymers, again with PEG ranging from 5-25%. U.S. Patent No. 4,526,938 to Churchill et al. (1985) described noncrosslinked

25   materials with MW in excess of 5,000, based on similar compositions with PEG; although these materials are not water soluble. Cohn et al. (1988) *J. Biomed. Mater. Res.* 22:993-1009 described PLA-PEG copolymers that swell in water up to 60%; these polymers also are not soluble in water, and are not crosslinked. The features that are common to these materials is that

30   they use both water-soluble polymers and degradable polymers, and that they are insoluble in water, collectively swelling up to about 60%.

U.S. Patent No. 5,410,016 issued on April 25, 1995 to Hubbell, et al., describes materials which are based on polyethylene glycol (PEG), because of its high biocompatible and thromboresistant nature, with short polylactide extensions to impart biodegradation and acrylate termini to allow rapid

5   photopolymerization without observable heat production. These materials are readily modified to produce hydrogels which release or produce NO.

The polymerizable regions are separated by at least one degradable region to facilitate uniform degradation *in vivo*. There are several variations of these polymers. For example, the polymerizable regions can be attached

10   directly to degradable extensions or indirectly via water soluble nondegradable sections so long as the polymerizable regions are separated by a degradable section. For example, if the macromer composition contains a simple water soluble region coupled to a degradable region, one polymerizable region may be attached to the water soluble region and the

15   other attached to the degradable extension or region. In another embodiment, the water soluble region forms the central core of the macromer composition and has at least two degradable regions attached to the core. At least two polymerizable regions are attached to the degradable regions so that, upon degradation, the polymerizable regions, particularly in the

20   polymerized gel form, are separated. Conversely, if the central core of the macromer composition is formed by a degradable region, at least two water soluble regions can be attached to the core and polymerizable regions /can be attached to each water soluble region. The net result will be the same after gel formation and exposure to *in vivo* degradation conditions.

25   In another embodiment, the macromer composition has a water soluble backbone region and a degradable region affixed to the macromer backbone. At least two polymerizable regions are attached to the degradable regions, so that they are separated upon degradation, resulting in gel product dissolution. In a further embodiment, the macromer backbone is formed of a

30   nondegradable backbone having water soluble regions as branches or grafts attached to the degradable backbone. Two or more polymerizable regions are attached to the water soluble branches or grafts. In another variation, the

backbone may be star shaped, which may include a water soluble region, a biodegradable region or a water soluble region which is also biodegradable. In this general embodiment, the star region contains either water soluble or biodegradable branches or grafts with polymerizable regions attached

5   thereto. Again, the polymerizable regions must be separated at some point by a degradable region.

Polymerizable groups *may be N. M*

The polymerizable regions ~~are preferably~~ polymerizable by photoinitiation by free radical generation, most preferably in the visible or

10 long wavelength ultraviolet radiation. The preferred polymerizable regions are acrylates, diacrylates, oligoacrylates, dimethacrylates, oligomethoacrylates, or other biologically acceptable photopolymerizable groups. A preferred tertiary amine is triethanol amine.

Useful photoinitiators are those which can be used to initiate by free

15 radical generation polymerization of the macromers without cytotoxicity and within a short time frame, minutes at most and most preferably seconds. Preferred dyes as initiators of choice for LWUV initiation are ethyl eosin, 2,2-dimethoxy-2-phenyl acetophenone, other acetophenone derivatives, and camphorquinone. In all cases, crosslinking and polymerization are initiated

20 among copolymers by a light-activated free-radical polymerization initiator such as 2,2-dimethoxy-2-phenylacetophenone or a combination of ethyl eosin (10^{-4}-10^{-2} milliM) and triethanolamine (0.001 to 0.1 M), for example.

The choice of the photoinitiator is largely dependent on the photopolymerizable regions. For example, when the macromer includes at

25 least one carbon-carbon double bond, light absorption by the dye causes the dye to assume a triplet state, the triplet state subsequently reacting with the amine to form a free radical which initiates polymerization. Preferred dyes for use with these materials include eosin dye and initiators such as 2,2-dimethyl-2-phenylacetophenone, 2-methoxy-2-phenylacetophenone, and

30 camphorquinone. Using such initiators, copolymers may be polymerized *in situ* by long wavelength ultraviolet light or by laser light of about 514 nm, for example.

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Initiation of polymerization is accomplished by irradiation with light at a wavelength of between about 200-700 nm, most preferably in the long wavelength ultraviolet range or visible range, 320 nm or higher, most preferably about 514 nm or 365 nm.

5   There are several photooxidizable and photoreducible dyes that may be used to initiate polymerization. These include acridine dyes, for example, acriblarine; thiazine dyes, for example, thionine; xanthine dyes, for example, rose bengal; and phenazine dyes, for example, methylene blue. These are used with cocatalysts such as amines, for example, triethanolamine; sulphur

10   compounds, for example, RSO_2R'; heterocycles, for example, imidazole; enolates; organometallics; and other compounds, such as N-phenyl glycine. Other initiators include camphorquinones and acetophenone derivatives.

Thermal polymerization initiator systems may also be used. Such systems that are unstable at 37°C and would initiate free radical

15   polymerization at physiological temperatures include, for example, potassium persulfate, with or without tetramethyl ethylenediamine; benzoylperoxide, with or without triethanolamine; and ammonium persulfate with sodium bisulfite.

Other initiation chemistries may be used besides photoinitiation.

20   These include, for example, water and amine initiation schemes with isocyanate or isothiocyanate containing macromers used as the polymerizable regions.

Preferred Embodiments

In the preferred embodiment, the polymeric materials are a

25   biodegradable, polymerizable and at least substantially water soluble macromer composition. The first macromer includes at least one water soluble region, at least one NO carrying region and at least one free radical-polymerizable region. The second macromer includes at least one water soluble region and at least two free radical polymerizable regions. The

30   regions can, in some embodiments, be both water soluble and biodegradable. The macromer composition is polymerized by exposure of the polymerizable regions to free radicals generated, for example, by photosensitive chemicals

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and dyes.

Examples of these macromers are PVA or PEG-oligoglycolyl-acrylates. The choice of appropriate end caps permits rapid polymerization and gelation. Acrylates are preferred because they can be polymerized using

5   several initiating systems, e.g., an eosin dye, by brief exposure to ultraviolet or visible light. A poly(ethyleneglycol) or PEG central structural unit (core) is preferred on the basis of its high hydrophilicity and water solubility, accompanied by excellent biocompatibility. A short oligo or poly(α-hydroxy acid), such as polyglycolic acid, is selected as a preferred chain

10 extension because it rapidly degrades by hydrolysis of the ester linkage into glycolic acid, a harmless metabolite. Although highly crystalline polyglycolic acid is insoluble in water and most common organic solvents, the entire macromer composition is water-soluble and can be rapidly gelled into a biodegradable network while in contact with aqueous tissue fluids.

15 Such networks can be used to entrap and homogeneously disperse water-soluble drugs and enzymes and to deliver them at a controlled rate. Further, they may be used to entrap particulate suspensions of water-insoluble drugs. Other preferred chain extensions are polylactic acid, polycaprolactone, polyorthoesters, and polyanhydrides. Polypeptides may also be used. Such

20 "polymeric" blocks should be understood to include timeric, dimeric, and oligomeric blocks.

PVA contains many pendant hydroxyl groups. These hydroxyl groups are easily reacted to form side chains such as various crosslinking agents and nitric oxide donors. PVA is water soluble and has excellent

25 biocompatiblity. Modification of PVA to attach methacrylate groups via a diacetal bond with the pendant hydroxyl groups and addition of an appropriate photoinitiator enables the PVA to be photopolymerized to form hydrogels under long wavelength UV light. In another preferred embodiment, the hydrogel is formed from modified polyvinyl alcohol (PVA)

30 macromers, such as those described in U.S. Patent Nos. 5,508,317, 5,665,840, 5,849,841, 5,932,674, 6,011,077, 5,939,489, and 5,807,927. The macromers disclosed in U.S. Patent No. 5,508,317, for example, are PVA prepolymers

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modified with pendant crosslinkable groups, such as acrylamide groups containing crosslinkable olefinically unsaturated groups. These macromers can be polymerized by photopolymerization or redox free radical polymerization, for example. The starting polymers are, in particular,

5   derivatives of polyvinyl alcohol or copolymers of vinyl alcohol that contain, for example, a 1,3-diol skeleton. The crosslinkable group or the further modifier can be bonded to the starting polymer skeleton in various ways, for example through a certain percentage of the 1,3-diol units being modified to give a 1,3-dioxane, which contains a crosslinkable radical, or a further

10   modifier in the 2-position. Another possibility is for a certain percentage of hydroxyl groups in the starting polymer to be esterified by means of an unsaturated organic acid, these ester-bonded radicals containing a crosslinkable group. The hydrophobicity of these macromers can be increased by substituting some of the pendant hydroxyl groups with more

15   hydrophobic substituents. The properties of the macromers, such as hydrophobicity, can also be modified by incorporating a comonomer in the macromer backbone. The macromers can also be formed having pendant groups crosslinkable by other means.

B.   NO groups or Modulating Compounds

20   A number of molecules that produce NO under physiological conditions (NO donors) have been identified and evaluated both *in vitro* and *in vivo,* including S-nitrosothiols, organic nitrates, and complexes of NO with nucleophiles. L-arginine is a NO donor, since L-arginine is a substrate for NO synthase, and thus administration of L-arginine increases endogenous

25   NO production and elicits responses similar to those caused by NO donors in most cases. Other NO donors include molsidomine, CAS754, SPM-5185, and SIN-1. Other compounds capable of producing and/or donating NO may also be used. These include organic nitrates, nitrosylating compounds, nitrosoesters, and L-arginine.

30   The molecules which produce NO, or release or generate NO, are preferably attached to regions containing nucleophiles and/or thiols such as S-nitrosothiols capable of forming a complex with NO.

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C.   Prophylactic, Therapeutic and Diagnostic Agents

The polymeric materials can also be used for drug delivery, preferably localized release of prophylactic, therapeutic or diagnostic agents at the site where the materials are needed, although the polymeric materials

5   can be loaded with agent to be released systemically. These agents include proteins or peptides, polysaccharides, nucleic acid molecules, and simple organic molecules, both natural and synthetic. Representative materials include antibiotics, antivirals, and antifungal drugs, anti-inflammatories (steroidal or non-steroidal), hormones, growth factors, cytokines, neuroactive

10   agents, vasoconstrictors and other molecules involved in the cardiovascular responses, enzymes, antineoplastic agents, local anesthetics, antiangiogenic agents, antibodies, drugs affecting reproductive organs, and oligonucleotides such as antisense oligonucleotides. Diagnostic materials may be radioactive, bound to or cleave a chromogenic substrate, or detectable by ultrasound, x-

15   ray, mri, or other standard imaging means.

These agents can be mixed with macromer prior to polymerization, applied into or onto the polymer, or bound to the macromer prior to or at the time of polymerization, either covalently or ionically, so that the agent is released by degradation (enzymatic or hydrolytic) or diffusion at the site

20   where the polymer is applied.

II.   Methods of Use

A.   Coatings; Films; Microparticles

Although described primarily with respect to *in vivo* treatment, it is apparent that the polymeric materials described herein can be used in cell

25   culture, on cell culture substrates, or as coatings on medical implants or devices such as stents or catheters, or formed using standard techniques into microparticles or other types of formulations which may be used in or administered to a patient.

B.   Therapeutic Applications

30   Polymeric materials capable of releasing physiological amounts of NO for prolonged periods of time can be applied to sites on or in a patient in need of treatment thereof. Representative disorders or conditions that can be

treated with NO include restenosis, thrombosis, asthma, wound healing, arthritis, and penile or female erectile dysfunction. The material is typically applied as a macromer solution and polymerized *in situ*, although polymerization can be initiated prior to application.

5   *Wound Healing*

The formulations are particularly useful for treatment of ~~wounds,~~ ~~such as ulcers and burns.~~ ... all types of wounds, including burns, surgical wounds, and open leg and foot wounds. There are generally three types of open leg wounds, termed ulcers: venous stasis ulcers, generally seen in
10   sedentary elderly people when blood flow to the leg becomes sluggish; decubitus ulcers, also termed pressure sores or bed sores, which occurs most often in people who are bedridden and are unable to frequently change position; and diabetic foot ulcers, caused by poor blood circulation to the feet. Due to the aging of the population, there will likely be a greater
15   demand for effective and user friendly wound treatments in the near future.

The term "wound" as used herein refers to all types of tissue injuries, including those inflicted by surgery and trauma, including burns, as well as injuries from chronic or acute medical conditions, such as atherosclerosis or diabetes.

20   *Treatment of Restenosis*

A preferred application is a method of reducing the effects of restenosis on post-surgical patients. The method includes coating the surface within an artery with an aqueous solution of light-sensitive free radical polymerizable initiator and a number of macromers. The coated artery is
25   subjected to a Xenon arc laser inducing polymerization of the macromers. As the newly polymerized macromer composition is formed, the physiological conditions within the artery will induce the release of NO. This release will be strictly localized for prolonged periods of time.

Prevention of Surgical Adhesions.

30   A preferred application is a method of reducing formation of adhesions after a surgical procedure in a patient. In one embodiment the method includes coating damaged tissue surfaces in a patient with an

aqueous solution of a light-sensitive free-radical polymerization initiator and a macromer solution as described above. The coated tissue surfaces are exposed to light sufficient to polymerize the macromer. The light-sensitive free-radical polymerization initiator may be a single compound (e.g., 2,2-

5   dimethoxy-2-phenyl acetophenone) or a combination of a dye and a cocatalyst (e.g., ethyl eosin and triethanol amine).

Tissue Adhesives.

Another use of the polymers is in a method for adhering tissue surfaces in a patient. In one embodiment the macromer is mixed with a

10   photoinitiator or photoinitiator/cocatalyst mixture to form an aqueous mixture and the mixture is applied to a tissue surface to which tissue adhesion is desired. The tissue surface is contacted with the tissue with which adhesion is desired, forming a tissue junction. The tissue junction is then irradiated until the macromers are polymerized.

15   *Tissue Coatings.*

In a particularly preferred application of these macromers, an ultrathin coating is applied to the surface of a tissue, most preferably the lumen of a tissue such as a blood vessel. One use of such a coating is in the treatment or prevention of restenosis, abrupt reclosure, or vasospasm after

20   vascular intervention. An initiator is applied to the surface of the tissue, allowed to react, adsorb or bond to tissue, the unbound initiator is removed by dilution or rinsing, and the macromer solution is applied and polymerized. This method is capable of creating uniform polymeric coating of between one and 500 microns in thickness, most preferably about twenty microns,

25   which does not evoke thrombosis or localized inflammation.

Tissue Supports.

The polymeric materials can also be used to create tissue supports by forming shaped articles within the body to serve a mechanical function. Such supports include, for example, sealants for bleeding organs, sealants for

30   bone defects and space-fillers for vascular aneurisms. Further, such supports can include strictures to hold organs, vessels or tubes in a particular position for a controlled period of time.

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Controlled drug delivery.

As noted above, the polymeric materials can be use as carriers for biologically active materials such as therapeutic, prophylactic or diagnostic agents, including hormones, enzymes, antibiotics, antineoplastic agents, and

5   cell suspensions. The polymeric material may be used to temporarily preserve functional properties of an agent to be released, as well as provide prolonged, controlled release of the agent into local tissues or systemic circulation.

In a variation of the method for controlled drug delivery in which

10   agent is mixed with the macromer solution then polymerized *in situ*, the macromers are polymerized with the biologically active materials to form microspheres or nanoparticles containing the biologically active material. The macromer, photoinitiator, and agent to be encapsulated are mixed in an aqueous mixture. Particles of the mixture are formed using standard

15   techniques, for example, by mixing in oil to form an emulsion, forming droplets in oil using a nozzle, or forming droplets in air using a nozzle. The suspension or droplets are irradiated with a light suitable for photopolymerization of the macromer.

These materials are particularly useful for controlled drug delivery of

20   hydrophilic materials, since the water soluble regions of the polymer enable access of water to the materials entrapped within the polymer. Moreover, it is possible to polymerize the macromer composition containing the material to be entrapped without exposing the material to organic solvents. Release may occur by diffusion of the material from the polymer prior to degradation

25   and/or by diffusion of the material from the polymer as it degrades, depending upon the characteristic pore sizes within the polymer, which is controlled by the molecular weight between crosslinks and the crosslink density. Deactivation of the entrapped material is reduced due to the immobilizing and protective effect of the gel and catastrophic burst effects

30   associated with other controlled-release systems are avoided. When the entrapped material is an enzyme, the enzyme can be exposed to substrate while the enzyme is entrapped, provided the gel proportions are chosen to

allow the substrate to permeate the gel. Degradation of the polymer facilitates eventual controlled release of free macromolecules in vivo by gradual hydrolysis of the terminal ester linkages.

III.   Examples

5   As demonstrated by examples 1-3, three classes of NO-producing, PEG-based polymers have been synthesized and their NO release rate constants determined in vitro under physiological conditions. The biological response to appropriate materials has been evaluated in vitro using cultured smooth muscle cells and endothelial cells and in vivo using a rat carotid

10   artery injury model that resembles restenosis in man. The materials include BAB block copolymers of polyethylene glycol (A) with polycysteine (B) that are subsequently reacted with $NaNO_2$ to form S-nitrosothiols, BAB block copolymers of polyethylene glycol ("PEG") (A) and diethylenetriamine ("DETA") (B) that are subsequently reacted with NO gas to form

15   nucleophile/NO complexes, and BAB block copolymers of polyethylene glycol (A) and polylysine (B) that are subsequently reacted with NO gas to form nucleophile/NO complexes. All polymers are further terminated with reactive acrylate groups to allow rapid photopolymerization in situ.

Such materials would be expected to have good biocompatibility,

20   provided that a water soluble, biocompatible polymer such as PEG comprises the bulk of the material and has a sufficiently high molecular weight, and to slowly biodegrade due to the presence of two ester bonds and two amide bonds in each polymer chain. These three materials were selected as they are expected to have vastly different release kinetics: nucleophile/NO

25   complexes have been shown to release NO for up to 5 weeks (Smith et al., 1996), while the half-life of S-nitrosocysteine is 0.023 hours (Mathews et al., 1993). The amount of NO produced by these copolymers may be tailored by altering the ratio of polyethylene glycol (PEG) to cysteine or lysine.

An advantage of these macromer compositions are that they can be

30   polymerized rapidly in an aqueous surrounding. Precisely conforming, semi-permeable, biodegradable films or membranes can thus be formed on tissue in situ to serve as biodegradable barriers, as carriers for living cells or other

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biologically active materials, and as surgical adhesives. The polymer shows

excellent biocompatibility, as seen by a minimal fibrous overgrowth on

implanted samples. Hydrogels for the models were gelled in situ from

water-soluble precursors by brief exposure to long wavelength ultraviolet

5   (LWUV) light, resulting in formation of an interpenetrating network of the

hydrogel with the protein and glycosaminoglycan components of the tissue.

      As demonstrated by examples 4 and 5, three types of PVA hydrogels

were made and demonstrated release of NO and incorporated drug (bFGF):

PVA-NH$_2$-NO hydrogels; PVA-Cys-NO hydrogels; PVA-NO-bFGF

10   hydrogels. The results are similar to those for the PEG based hydrogels.

Example 1:   **Synthesis of PEG-Cys Macromers**

      As shown in Figure 1, an acryloyl-PEG-CYSNO polymer was

formed by first reacting polyethylene glycol N-hydroxysuccinimide

monoacrylate (ACRL-PEG-NHS, MW 3400, commercially available from

15   Shearwater Polymers, Huntington, AL) with L-cysteine at an 1:2 molar ratio

in 50 mM sodium bicarbonate buffer (pH 8.5) for 2 hours; the product was

then dialyzed in a cellulose ester membrane (Molecular weight cutoff 500,

Spectrum Labs, Laguna Hills, CA) in diH$_2$O, and lyophilized. Analysis of

the acryloyl-PEG-Cys copolymer was performed using gel permeation

20   chromatography (GPC) with an evaporative light scattering detector and a

UV detector at 260 nm (Polymer Laboratories, Amherst, MA). Successful

synthesis of acryloyl-PEG-Cys was determined by a shift in the position of

the peak from the evaporative light scattering detector. The copolymer was

then reacted with an equimolar amount of NaNO$_2$ at pH 2 and 37°C for 20

25   minutes to form S-nitrosocysteine. Conversion of thiol groups to S-

nitrosothiols was measured using the Ellman's assay (Hermanson, 1995).

After adjusting the pH of the solution to 7.4, the acryloyl-PEG-CYSNO

polymer was incorporated into photopolymerizable hydrogels by mixing

with PEG-diacrylate (MW 3400) at a 1:10 molar ratio in aqueous solution

30   with 1500 ppm 2,2-dimethoxy-2-phenyl acetophenone as a long wavelength

ultraviolet initiator. 0.15% N-vinylpyrrolidone was present in this mixture

as it was used as a solvent for the photoinitiator. Exposure to UV light (365

nm, 10 mW/cm^2) was used to crosslink the polymer, resulting in conversion

to a hydrogel (Sawhney et al., ~~1993). Production of NO by the hydrogels~~

~~was quantified using the Griess assay.~~ *μg(s)* *Lys5-NO*

* amd Hydrogels*

Example 2:   Synthesis of PEG-~~Lys~~-Macromers.

As shown in Figure 2, for acryloyl-PEG-Lys$_5$-NO hydrogels, a

copolymer of ACRL-PEG-NHS (MW 3400, Shearwater Polymers) and

poly-L-lysine (DP=5) was synthesized by reacting at an equimolar ratio in

50 mM sodium bicarbonate (pH 8.5).   The resultant copolymer was

analyzed via GPC, then dissolved in water and reacted with NO gas in an

evacuated vessel, thus forming NO-nucleophile complexes with the amine

groups on the lysine side groups. The extent of conversion of amine groups

to NO-nucleophile complexes was measured using the ninhydrin assay, and

crosslinked hydrogels were formed as described above in Example 1. *Hy drogels*

Example 3:   Synthesis of DETA-NO-~~nucleophile complex hydrogels.~~ *macromers and Hy*

Diethylenetriamine (DETA, Aldrich, Milwaukee, WI) was reacted

with ACRL-PEG-NHS (MW 3400, Shearwater Polymers) in 50 mM sodium

bicarbonate buffer (pH 8.5) at an equimolar ratio, lyophilized, and analyzed

via GPC as described above. The copolymer was then dissolved in water

and exposed to NO gas to form NO-nucleophile complexes as described for

PEG-Lys$_5$-NO and assayed for amine content using the ninhydrin assay. The

PEG-DETA-NO was lyophilized and then photopolymerized as described

above to form hydrogels, as shown in Figure 3. *macromers and Hydrogels*

Example 4:   Synthesis of PVA-NH$_2$-NO ~~hydrogels~~

Poly(vinyl alcohol) (Hoechst, Mowiol 4-88) was dissolved in diH$_2$0

and warmed to 95°C in a round bottom flask under continuous stirring. After

one hour, the solution was cooled to room temperature, and a crosslinkable

acetal group, methacrylamidoacetaldehyde dimethyl acetal (NAAADA) was

added. The amine acetal, gamma-aminobutyraldehyde diethyl acetal, was

also added, and the mixture was acidified using glacial acetic acid and 37%

hydrochloric acid. The mixture was allowed to stir at room temperature for

nine hours, after which the pH was adjusted to pH 3.6 using triethylamine.

In order to purify the polymer, the solution was then diafiltered through a

MW 3000 cellulose membrane against diH$_2$O at 6.5 times the volume of polymer solution. The polymer concentration was adjusted to 22% w/v using diafiltration, and the pH was adjusted to 7.4 with 1N NaOH. The amine concentration of the polymer was determined using the ninhydrin

5   assay.

In order to form the NO donor bound to the PVA-NH$_2$, the neutralized amine-modified polymer was placed in a round bottom flask with stopcock. The flask was evacuated and filled with nitric oxide gas until the desired conversion of amines to NO nucleophile complexes was achieved.

10   Photocrosslinked hydrogels were formed from the PVA-NH$_2$-NO by adding 0.1% ~~Irgacure~~ _Irgacure_ 2959 (Ciba-Geigy) photoinitiator (based on total solution volume) and then exposing to UV light (2 mW/cm^2, 365 nm) for 30 seconds. Addition of the photoinitiator brings the final polymer concentration to 20% w/v.   _Mackomers and Hydrogels_

15   **Example 5:   Synthesis of PVA-Cys-NO ~~hydrogels~~**

PVA-NH$_2$ was synthesized as described above. The amine terminus of cysteine was acetylated using acetic anhydride, and the carboxyl end of the cysteine was coupled to the PVA-NH$_2$ using water-soluble EDAC chemistry. The resulting PVA-Cys was then purified using diafiltration and

20   brought to a concentration of 22% w/v. PVA-Cys-NO was formed by adding sodium nitrite at an equimolar amount to cysteine residues, adjusting the pH to 2, and incubating at 37°C for 15 minutes. The extent of reaction of cysteine to Cys-NO was assayed using both the Ellman's and Griess reactions. The photoinitiator, 2,2-methyl-2-phenylacetophenone was

25   dissolved in N-vinylpyrrolidone at a concentration of 600 mg/ml and added to the polymer solution (0.1% based on total solution volume). The polymer was then crosslinked under UV light for 30 seconds and placed in HEPES buffered saline, pH 7.4, 37°C.   _Synthesis of_   _Hydrogels_

_/_   **Example 6:   ~~Release of bFGF~~ from PVA-NO-bFGF ~~hydrogels~~.**

30   For PVA-NO-bFGF hydrogels, the above procedure was used to make the PVA-NO polymer. Immediately prior to exposure to UV light, 25

μg/ml bFGF was added to the polymer solution and mixed well. Gels were crosslinked as described earlier and stored in HEPES buffered saline, pH 7.4, 37°C. ~~Release of bFGF was~~ quantified using the BCA assay (Pierce Chemicals), ~~and NO release was assayed using the Griess reaction.~~ *No Release Hormones*

Example 7: ~~NO-release Rates~~ from acryloyl-PEG-Lys₅-NO hydrogels

Following preparation and photopolymerization of the NO-releasing materials as described above, the hydrogels were weighed and stored in HEPES buffered saline, pH 7.4, at 37°C. Aliquots of the buffer were removed at each time point and replaced with fresh buffer. The samples from each time point were then analyzed for nitrite content using a colorimetric assay based on the Griess reaction.

NO release kinetics of hydrogels stored in buffer at various pH levels were also investigated in order to explore possible storage conditions for the hydrogels. At acidic pH levels, release of NO from the hydrogels was significantly inhibited.

NO release from acryloyl-PEG-Lys₅-NO hydrogels is shown in Figure 4.

NO release from acryloyl -PEG-DETA-NO hydrogels is shown in Figure 5. *CYS-NO*

NO release from acryloyl-PEG-~~CYSNO~~ hydrogels is shown in Figure 6.

No and bFGF Release Hydrogels

Example 8: ~~NO-release Rates~~ from PVA-NO-bFGF hydrogels

The release of NO release from PVA-NO-bFGF hydrogels was determined in the same manner as Example 7 and is shown in ~~Figure 7.~~ *are changed.*

~~Figures 12a and 12b, respectively, show the temporal release of NO and a growth factor, bFGF, over time from PVA-NO-bFGF hydrogels.~~ Release of NO continues for well over 12 hours, while the growth factor is completely released within the first 5 hours.

Example 9: **Effects of NO-releasing Macromers on Cultured Smooth Muscle Cells: Proliferation and Viability**

In order to assess the potential of a material for the reduction of smooth muscle cell proliferation after vascular injury, cultured smooth

muscle cells were grown in the presence of NO-releasing materials, and the effects of those materials on the cells evaluated. Smooth muscle cells isolated from Wistar-Kyoto rats (passage 11-15, provided by T. Scott-Burden) were cultured in Minimum Essential Medium supplemented with

5   10% FBS, 2 mM L-glutamine, 500 units penicillin, and 100 mg/L streptomycin, at 37°C in a 5% CO_2 environment. The cells were seeded into 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at a density of 10,000 cells/cm^2. NO donors in either soluble or hydrogel form were added to the media in the wells one day after seeding. At 4 days

10   culture, cell numbers were determined by preparing single cell suspensions with trypsin and counting three samples from each group using a Coulter counter (Multisizer #0646, Coulter Electronics, Hialeah, FL).

The effects of NO donors in solution on the proliferation of SMCs were first investigated by performing a NO dose response curve, whereupon

15   cells were cultured with a range of NO donor concentrations (1 µM - 10 mM) in order to identify appropriate dosages for hydrogel studies. NO-nucleophile complexes (Lys-NO and DETA-NO) were formed by reacting either L-lysine or DETA with NO gas in water for 24 hours. Soluble Cys-NO was synthesized by reacting an equimolar amount of L-cysteine with

20   $NaNO_2$ at pH 2 and 37°C for 20 minutes. All NO donor solutions were adjusted to pH 7.4 prior to addition to cell cultures.

Smooth muscle cell proliferation in the presence of NO-producing and control hydrogels was then investigated using the optimal NO dose determined above. Hydrogels containing acryloyl-PEG-Lys-NO, acryloyl-

25   PEG-DETA-NO, and acryloyl-PEG-CYSNO were formed as described above, except that the gel solutions were sterile filtered through 0.2 µm syringe filters (Gelman Sciences, Ann Arbor, MI) prior to adding 2,2-dimethoxy-2-phenyl acetophenone. PEG-diacrylate hydrogels containing no NO donors were used as a control. The hydrogels were photopolymerized in

30   cell culture inserts (8 µm pore size, Becton Dickinson, Franklin Lakes, NJ) and placed in the media over the cultured cells.

/ All three hydrogel NO donors significantly inhibited SMC growth (p < 0.0001). The number of smooth muscle cells remained near that of the seeding density, which ranged from 10-15% of the final control cell number for all experiments.

5   Inhibition of SMC proliferation by acryloyl-PEG-Lys$_5$-NO hydrogels is shown in Figure 8A, compared to the macromer solution control shown in Figure 8B. Both significantly inhibited SMC proliferation.

Inhibition of SMC proliferation by acryloyl-PEG-DETA-NO-nucleophile complex hydrogels is shown in Figure 9A, compared to the

10   macromer solution control shown in Figure 9B. Both significantly inhibited SMC proliferation.   CYS-NO

Inhibition of SMC proliferation by acryloyl-PEG-CYSNO hydrogels is shown in Figure 10A, compared to the macromer solution control shown in Figure 10B. Both significantly inhibited SMC proliferation.   CYS-NO

15   Inhibition of SMC proliferation by acryloyl-PEG-CYSNO hydrogels, acryloyl-PEG-DETA-NO hydrogels, and acryloyl-PEG-Lys-NO hydrogels is compared to the control hydrogel in Figure 11. All of the NO hydrogels significantly inhibited SMC growth.

Example 5.10   Effects of NO-releasing Macromers on Platelet Adhesion

20   *in vitro.*

The effect of NO release on platelet adhesion was investigated to assess the potential of these materials for prevention of thrombosis. Blood was obtained from a healthy volunteer by venipuncture and anticoagulated with 10 U/ml heparin. Platelets and white blood cells were fluorescently

25   labeled with mepacrine at a concentration of 10 μM. A solution of 2.5 mg/ml collagen I in 3% glacial acetic acid in diH$_2$O was prepared and applied to glass slides for 45 minutes in a humidified environment at room temperature. Acryloyl-PEG-CYSNO and PEG-diacrylate hydrogels were prepared as described above and incubated with the labeled whole blood at

30   37°C for 30 minutes. The hydrogels were removed and the blood was then incubated with the collagen-coated glass slides (two per group) for 20 minutes at 37°C and then rinsed with HBS. Platelet counts per field of view

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at 40x were counted under a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY) in four randomly chosen areas per slide.

Photos of platelets which had been exposed to control PEG-diacrylate or acryloyl-PEG-C̶Y̶S̶N̶O̶ *CYS-NO* hydrogels demonstrate that exposure to the NO-releasing hydrogels inhibits platelet adhesion to thrombogenic surfaces. Glass slides coated with collagen were used as a thrombogenic surface to which platelets would normally adhere. When the blood was incubated with control PEG-diacrylate hydrogels, 69.25 ± 4.46 (mean $\pm$ SD) adherent platelets were observed per field of view. This number was reduced to 7.65 ± 6.16 platelets pre field of view when blood was pre-exposed to the acryloyl-PEG-C̶Y̶S̶N̶O̶ *CYS-NO* hydrogels ($p < 0.0001$).

Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art from the foregoing detailed description and accompanying figures. These methods and materials are intended to be encompassed by the following claims.